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<b>(21) International Application Number:</b> PCT/US96/01842 <b>(22) International Filing Date:</b> 9 February 1996 (09.02.96) <b>(30) Priority Data:</b> 08/386,452 10 February 1995 (10.02.95) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/386,452 (CIP) Filed on 10 February 1995 (10.02.95) <b>(71) Applicants (for all designated States except US):</b> PURDUE RE- SEARCH FOUNDATION [US/US]; Room 328, 1650 Engi- neering Administration Building, West Lafayette, IN 47907- 1650 (US). METHODIST HOSPITAL OF INDIANA, INC. [US/US]; 1701 North Senate Boulevard, P.O. Box 1367, In- dianapolis, IN 46206-1367 (US). ELI LILLY AND COM- PANY [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BADYLAK, Stephen, F. [US/US]; 2610 Nottingham Place, West Lafayette, IN 47906 (US). BODER, George [US/US]; 1008 Maple Turn Road,		Martinsville, IN 46151 (US). VOYTIK, Sherry [US/US]; 113 Red Cloud Trail #905, Lafayette, IN 47905 (US). DEMETER, Robert, J. [US/US]; 360 Cottonwood Drive, Mooresville, IN 46158 (US). CRITSER, John, K. [US/US]; 11341 Rolling Springs Drive, Carmel, IN 46033 (US). LIU, Chi [US/US]; 6045 Crows Nest Drive, Indianapolis, IN 46208 (US). <b>(74) Agents:</b> LAMMERT, Steven, R. et al.; Barnes & Thornburg, 1313 Merchants Bank Building, 11 South Meridian Street, Indianapolis, IN 46204 (US). <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> SUBMUCOSA AS A GROWTH SUBSTRATE FOR CELLS <b>(57) Abstract</b> <p>A cell culture growth substrate comprising submucosal tissue of a warm-blooded vertebrate and a method for culturing eukaryotic cells are described. Submucosal tissue used in accordance with the present invention supports the proliferation and differentiation of eukaryotic cells when said cells are contacted with submucosal tissue under conditions conducive to cell proliferation.</p>		

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SUBMUCOSA AS A GROWTH SUBSTRATE FOR CELLSField of the Invention

The present invention relates to the culturing of eukaryotic cells. More particularly, this invention is directed to a method for supporting the growth and tissue differentiation of eukaryotic cells *in vitro*, by contacting the eukaryotic cells with submucosal tissue of a warm-blooded vertebrate, under conditions conducive to eukaryotic cell proliferation.

Background and Summary of the Invention

Tissue culture allows the study *in vitro* of animal cell behavior in an investigator-controlled physiochemical environment. However, cellular morphology and metabolic activity of cultured cells are affected by the composition of the substrate on which they are grown. Presumably cultured cells function best (i.e. proliferate and perform their natural *in vivo* functions) when cultured on substrates that closely mimic their natural environment. Currently, studies *in vitro* of cellular function are limited by the availability of cell growth substrates that present the appropriate physiological environment for proliferation and development of the cultured cells.

The ability of complex substrates to support cell growth *in vitro* has been previously reported, and matrix products supporting such growth are commercially available. For example, Becton Dickinson currently offers two such products: Human Extracellular Matrix and MATRIGEL® Basement Membrane Matrix. Human Extracellular Matrix is a chromatographically partially purified matrix extract derived from human placenta and comprises laminin, collagen IV, and heparin sulfate proteoglycan. (Kleinman, HK et al., US Patent 4,829,000 (1989)) MATRIGEL® is a soluble basement membrane extract of the Engelbreth-Holm-Swarm

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(EHS) tumor, gelled to form a reconstituted basement membrane. Both of these matrix products require costly biochemical isolation, purification, and synthesis techniques and thus production costs are high.

5           The present invention is directed to the use of vertebrate submucosa-derived matrices as substrates for the growth and attachment of a wide variety of cell types. The collagenous matrices for use in accordance with the present invention comprise highly conserved collagens,  
10 glycoproteins, proteoglycans, and glycosaminoglycans in their natural configuration and natural concentration. The extracellular collagenous matrix for use in this invention is derived from submucosal tissue of a warm-blooded vertebrate. Submucosal tissue can be obtained from various  
15 sources, including intestinal tissue harvested from animals raised for meat production, including, for example, pigs, cattle and sheep or other warm-blooded vertebrates. This tissue can be used in either its natural configuration or in a comminuted or partially digested fluidized form.  
20 Vertebrate submucosal tissue is a plentiful by-product of commercial meat production operations and is thus a low cost cell growth substrate, especially when the submucosal tissue is used in its native layer sheet configuration.

          The submucosal cell growth substrates of this  
25 invention provide cells with a collagenous matrix environment *in vitro* resembling that found *in vivo*. The natural composition and configuration of submucosal tissue provides a unique cell growth substrate that promotes the attachment and proliferation of cells.

30           Accordingly, one object of the present invention is to provide a relatively inexpensive cell culture growth substrate that promotes or induces growth and differentiation of cells cultured *in vitro*.

          Another object of this invention is to provide a  
35 method for improving cell proliferation in cell/tissue

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culture by using vertebrate submucosal tissue as a substrate for cell/tissue growth *in vitro*.

Another object of this invention is to provide a cell culture composition including a proliferating cell  
5 population in contact with submucosal tissue of a warm-blooded vertebrate and a nutrient medium for support of the growth of said cell population.

Still a further object of this invention is to provide a model system for studying tumor cell growth. The  
10 model system comprises a proliferating tumor cell population in contact with submucosal tissue of a warm-blooded vertebrate and a nutrient medium. The submucosal tissue substrates provide an *in vitro* environment resembling that found *in vivo* and thus in accordance with  
15 the present invention serve as a model system for the study of tumor cell growth characteristics. Such a model system would allow detailed characterization of the cellular and molecular processes involved in tumor cell growth and invasion of nontumor tissues.

20 One other object of this invention is to provide a culture system (referred to herein as an "invasion chamber") and a method for analyzing the invasive growth properties of eukaryotic cells. The invasion chamber comprises first and second chambers separated by a  
25 substrate interface, wherein the substrate interface comprises submucosa tissue. Cells are cultured in the invasion chamber by seeding the cells directly onto the submucosal substrate interface, filling the first and second chambers with nutrient media to support the growth  
30 of cells, and culturing the cells under conditions conducive to the growth of the cells. The cells can be cultured under optimal growth conditions to study general cell growth characteristics, or the growth conditions can be varied to study the response of cells to those changes  
35 in growth conditions.

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In one embodiment, tumor cells are cultured in contact with the substrate interface of the invasion chamber under varying growth conditions to investigate the growth and invasive properties of the tumor cells and their response to various growth conditions. The mucosal tissue substrate and the tumor cell population on the substrate can then be examined using standard histological means.

It has been reported that compositions comprising submucosal tissue of the intestine of warm-blooded vertebrates can be used as tissue graft materials in sheet or fluidized form. U.S. Patent No. 4,902,508 describes tissue graft compositions that are characterized by excellent mechanical properties, including high compliance, a high burst pressure point, and an effective porosity index. These properties allow such compositions to be used for vascular and connective tissue graft constructs. When used in such applications the preferred graft constructs serve as a matrix for the *in vivo* regrowth of the tissues replaced by the graft constructs. U.S. Patent No. 5,275,826 describes use of fluidized forms of vertebrate submucosal tissues as injectable or implantable tissue grafts.

An additional object of the present invention is to enhance or expand the functional properties of vertebrate submucosal tissues as an implantable or injectable tissue graft construct by seeding the submucosal tissue *in vitro* with preselected or predetermined cell types prior to implanting or injecting the graft construct into the host.

#### Brief Description of the Drawings

Fig. 1 is a perspective exploded assembly view of an invasion chamber in accordance with the present invention; and

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Fig. 2 is a cross-sectional view of an assembled invasion chamber showing the upper body and interface substrate in engagement with the base.

5 Detailed Description of the Preferred Embodiments

There is provided in accordance with this invention a method and composition for supporting the proliferation and inducing tissue differentiation of eukaryotic cells cultured in vitro. Generally the method  
10 comprises the step of contacting eukaryotic cells, in vitro, with a vertebrate submucosa-derived collagenous matrix under conditions conducive to eukaryotic cell growth. The term "contacting" as used herein with reference to cell culture is intended to include both  
15 direct and indirect contact, for example in fluid communication, of the submucosal tissue and the cultured cells. The term "conditions conducive to eukaryotic cell growth" as used herein refers to the environmental conditions, such as sterile technique, temperature and  
20 nutrient supply, that are considered optimal for eukaryotic cell growth under currently available cell culture procedures. Although optimum cell culture conditions used for culturing eukaryotic cells depend somewhat on the particular cell type, cell growth conditions are generally  
25 well known in the art. However a number of differentiated cell types are still considered difficult to culture (i.e. islets of Langerhans, hepatocytes, chondrocytes, osteoblasts, etc.).

The collagenous matrix component of the present cell  
30 culture substrate is derived from vertebrate submucosa and comprises naturally associated extracellular matrix proteins, glycoproteins and other factors. Preferably the collagenous matrix comprises intestinal submucosal tissue of a warm-blooded vertebrate. The small intestine of warm-

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blooded vertebrates is a particularly preferred source of the cell culture substrate for use in this invention.

Suitable intestinal submucosal tissue typically comprises the tunica submucosa delaminated from the tunica muscularis and at least the luminal portion of the tunica mucosa. In one preferred embodiment of the present invention the intestinal submucosal tissue comprises the tunica submucosa and basilar portions of the tunica mucosa including the lamina muscularis mucosa and the stratum compactum which layers are known to vary in thickness and in definition dependent on the source vertebrate species.

The preparation of submucosal tissue for use in accordance with this invention is described in U.S. Patent No. 4,902,508, the disclosure of which is expressly incorporated herein by reference. A segment of vertebrate intestine, preferably harvested from porcine, ovine or bovine species, but not excluding other species, is subjected to abrasion using a longitudinal wiping motion to remove the outer layers, comprising smooth muscle tissues, and the innermost layer, i.e., the luminal portion of the tunica mucosa. The submucosal tissue is rinsed with saline and optionally sterilized; it can be stored in a hydrated or dehydrated state. Lyophilized or air dried submucosa tissue can be rehydrated and used in accordance with this invention without significant loss of its cell proliferative activity.

The graft compositions of the present invention can be sterilized using conventional sterilization techniques including glutaraldehyde tanning, formaldehyde tanning at acidic pH, propylene oxide treatment, gas plasma sterilization, gamma radiation, electron beam, peracetic acid sterilization. Sterilization techniques which do not adversely affect the mechanical strength, structure, and biotropic properties of the submucosal tissue is preferred. For instance, strong gamma radiation may cause loss of



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strength of the sheets of submucosal tissue. Preferred sterilization techniques include exposing the graft to peracetic acid, 1-4 Mrads gamma irradiation (more preferably 1-2.5 Mrads of gamma irradiation) or gas plasma sterilization; peracetic acid sterilization is the most preferred sterilization method. Typically, the submucosal tissue is subjected to two or more sterilization processes. After the submucosal tissue is sterilized, for example by chemical treatment, the tissue may be wrapped in a plastic or foil wrap and sterilized again using electron beam or gamma irradiation sterilization techniques.

The submucosal tissue specified for use in accordance with this invention can also be in a fluidized form. Submucosal tissue can be fluidized by comminuting the tissue and optionally subjecting it to protease digestion to form a homogenous solution. The preparation of fluidized forms of submucosa tissue is described in U.S. Patent No. 5,275,826, the disclosure of which is expressly incorporated herein by reference. Fluidized forms of submucosal tissue are prepared by comminuting submucosa tissue by tearing, cutting, grinding, or shearing the harvested submucosal tissue. Thus pieces of submucosal tissue can be comminuted by shearing in a high speed blender, or by grinding the submucosa in a frozen or freeze-dried state to produce a powder that can thereafter be hydrated with water or a buffered saline to form a submucosal fluid of liquid, gel or paste-like consistency. The fluidized submucosa formulation can further be treated with a protease such as trypsin or pepsin at an acidic pH for a period of time sufficient to solubilize all or a major portion of the submucosal tissue components and optionally filtered to provide a homogenous solution of partially solubilized submucosa.

The viscosity of fluidized submucosa for use in accordance with this invention can be manipulated by

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controlling the concentration of the submucosa component and the degree of hydration. The viscosity can be adjusted to a range of about 2 to about 300,000 cps at 25° C. Higher viscosity formulations, for example, gels, can be prepared from the submucosa digest solutions by adjusting the pH of such solutions to about 6.0 to about 7.0.

Forming the basis of this invention it has been discovered that compositions comprising submucosal tissue can be used for supporting growth or proliferation of eukaryotic cells *in vitro*. Submucosal tissue can be used in accordance with this invention as a cell growth substrate in a variety of forms, including its native sheet-like configuration, as a gel matrix, as an addition for art-recognized cell/tissue culture media, or as coating for culture-ware to provide a more physiologically relevant substrate that supports and enhances the proliferation of cells in contact with the submucosal matrix. The submucosal tissue provides surfaces for cell adhesion and also induces cell differentiation. The submucosal tissue is preferably sterilized prior to use in cell culture applications, however nonsterile submucosal tissue can be used if antibiotics are included in the cell culture system.

In one preferred embodiment cells are seeded directly onto sheets of vertebrate submucosal tissue under conditions conducive to eukaryotic cell proliferation. The porous nature of submucosal tissue allows diffusion of cell nutrients throughout the submucosal matrix. Thus, for example, cells can be cultured on either the luminal or abluminal surface of the submucosal tissue. The luminal surface is the submucosal surface facing the lumen of the organ source and typically adjacent to an inner mucosa layer *in vivo* whereas the abluminal surface is the submucosal surface facing away from the lumen of the organ and typically in contact with smooth muscle tissue *in vivo*.

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Cells cultured on solid sheets of vertebrate submucosal tissue display a different growth pattern, and exhibit different interactions with the submucosal growth substrate, depending on which side of the submucosal sheet the cells are grown. Histological examination of tissue/cells cultured on intestinal submucosal tissue sheets in accordance with this invention reveals that cells that are seeded onto the abluminal surface not only grow/proliferate along the surface of the submucosal tissue, but they also more readily migrate into and proliferate within the submucosal tissue itself. The luminal surface comprises a more dense matrix than the abluminal side and thus cells are less likely to penetrate the luminal side. Cells that are seeded onto the luminal surface attach to the matrix but generally do not penetrate the surface. However certain cell types are capable of penetrating both the abluminal and luminal surfaces (eg squamous carcinoma cells and fibroblasts). In addition, certain cell types, such as fetal rat cells, when seeded on the luminal side proliferate to form a polylayer of cells. Cells of this polylayer can differentiate to perform functions characteristic of cells *in vivo* and indicative of their position in the polylayer.

In one embodiment in accordance with the present invention the submucosal cell substrates can be utilized to provide a model system for studying tumor cell growth. Tumor invasion of basement membranes is a crucial step in the complex multistage process which leads to the formation of metastases. Common features of the invasive process include: (a) attachment of the tumor cells to the basement membrane via cell surface receptors; (b) secretion of enzymes by the tumor cells that cause degradation of the adjacent extracellular matrix (ECM) structures; and (c) migration of the cells through the matrix components. Tumor cells cultured *in vitro* however, typically form a

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monolayer or polylayer of flattened cells that is not appropriate for studying the process of tissue invasion by tumor cells. However tumor cells cultured on the submucosa cell culture substrates of the present invention actively  
5 degrade the submucosal matrix components and migrate/invade the substrate matrix.

Cell culture substrates comprising submucosal tissue of warm blooded vertebrates can be used to culture various tumor cells types *in vitro* as a model of tumor cell  
10 growth characteristics. Such a model system allows investigation of the molecular mechanisms involved in tumor invasion and could ultimately lead to the development of novel anti-metastatic therapeutic strategies. In particular, such a model system would enable the analysis  
15 *in vitro* of the effect of various compounds, such as growth factors, anti-tumor agents, chemotherapeutics, antibodies, irradiation or other factors known to effect cell growth, on the growth characteristics of tumor cells.

To analyze *in vitro* the effect of varying cell  
20 growth conditions on the growth characteristics of tumor cells, tumor cells are seeded on a cell growth substrate comprising submucosal tissue of a warm-blooded vertebrate and provided a culture medium containing nutrients necessary to the proliferation of said cells. The seeded  
25 cells are then cultured under a preselected variable cell growth condition for varying lengths of time and then the mucosal tissue substrate, and the tumor cell population on the substrate are histologically examined. A variety of growth conditions can be investigated for their effect on  
30 tumor cell growth and/or invasion of cell matrices, including the presence or concentration of a tumor cell growth modifier compound, such as cytokines or cytotoxic agents, in the nutrient medium. Alternatively the selected growth condition may be the modification of environmental  
35 factors such as temperature, pH, electromagnetic radiation,

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or nutrient composition. The effect of the selected growth condition on the morphology and growth of tumor cells can then be assessed by histological analysis of control (tumor cells cultured in the absence of the selected growth condition) and test tumor cell cultures. In addition to standard histological techniques, the growth characteristics of the cultured cells can also be analyzed by labeling the cells using various methods including radioactive or fluorescent probes and culturing the labelled cells on the submucosa derived substrates.

Submucosal tissue, in both fluidized and sheet forms, can be utilized in conjunction with various tissue culture products to fashion *in vitro* culture systems for studying *in vitro* the invasive properties of tumor cells. For example, fluidized forms of submucosal tissue can be used to coat polycarbonate filters and then applied to a Boyden chamber-like device to create an invasion chamber. In addition, Blind Well chambers available through Neuroprobe, Inc. (Cabin John, Maryland) readily accommodate various configurations of submucosal tissue (e.g., intact submucosal tissue-coated polycarbonate filters, soluble submucosal tissue-coated polycarbonate filters, or intact submucosal tissue alone) for development of invasion chambers.

Invasion chambers are useful for the *in vitro* assessment of the invasive properties of cells. To date, Matrigel, a gelatinous extract of ECM from Englebreth-Holm-Swarm tumor cells is the most widely used ECM substrate for such invasion studies. However, this substrate is costly, difficult to manipulate, and represents reconstituted (not natural) extracellular matrix from neoplastic (not normal physiologic) tissue.

An invasion chamber in accordance with the present invention comprises an upper body defining a first chamber, a base defining a second chamber, and a substrate

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interface separating said first and second chambers, wherein the substrate interface comprises submucosal tissue of a warm-blooded vertebrate tissue. The upper body is biased against the base to hold the submucosa substrate interface between the first and second chambers. In use cells are seeded directly onto the substrate interface, and the first and second chambers are filled with nutrient media to support the growth of the cells under preselected cell growth conditions. The invasion chamber can be further provided with means for accessing the first and second chambers to supplement or modify nutrient or other cell growth conditions during use. In one embodiment the means for accessing the first and second chambers comprises a port with a removable plug that seals the port.

In one embodiment the invasion chamber comprises an upper body having an axially extending projection, a base having a cavity formed in the upper surface for receiving the axially extending projection, wherein the inner surface of the wall defining the cavity is provided with a rim, and a substrate interface formed to be inserted into the cavity of the base and seated on the rim. In one embodiment, the axially extending projection is an annular extension and the base cavity is cylindrically shaped. The axially extending projection and the wall defining the base cavity can also independently be polyhedral in shape, including square-shaped and other multi-sided configurations. The substrate interface comprises a layer of submucosal tissue and can be submucosal tissue in sheet form or a filter, screen, mesh membrane or their equivalents coated or layered with submucosal tissue in sheet or fluidized form. In one embodiment the substrate interface comprises submucosal tissue coated porous filters.

The substrate interface in cooperation with the cavity of the base and the axially extending projection of

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the upper body defines an upper and lower chamber. Upon insertion of the axially extending projection of the upper body into the base cavity, the portion of the substrate interface seated on the rim is pressed against the rim by the end portion of the axially extending projection of the upper body. Thus the substrate interface is secured between the rim and the axially extending projection. A washer or washers can be positioned between the rim and the substrate interface and/or between the substrate interface and the axially extending projection.

The biasing force compressing the substrate interface between the rim and the end portion of the axially extending projection can be provided by a set of springs or clamps. Alternatively, the axially extending projection of the upper body can be formed to, or provided with means for, frictionally engaging the inner surface of the wall defining the cavity of the base, so as to hold the axially extending projection against the substrate interface after insertion of the axially extending projection into the cavity of the base. In one embodiment the components of the invasion chamber are constructed from substantially transparent materials including glass and transparent plastics.

In the use of the invasion chamber, cell culture medium is first introduced into the cavity of the base. The substrate interface and the upper body are then inserted into the cavity of the base, and cell culture medium is introduced into the upper chamber. Cells are seeded onto the upper surface of the substrate interface in contact with the submucosal tissue layer, and the cells are cultured using standard techniques known to those skilled in the art. Typically, a chemoattractant is added to the lower chamber to promote invasion of the substrate interface by the cultured cells.

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After the cells are cultured for a predetermined length of time, standard histochemical techniques are used to assess the invasive properties of the cultured cells. Various stains, fluorescent markers, or radionucleides can be used to obtain quantitative as well as qualitative invasion data. The invasion chambers of the present invention can be used to assess the invasive potential of various cell types as well as a means for selective isolation of cells based upon their invasive potential.

An invasion chamber in accordance with one embodiment of the present invention is illustrated in Fig. 1. The invasion chamber 10 comprises an upper body 12, a base 14 and a substrate interface 16. Upper body 12 is provided with a cannular space 18 extending through upper body 12 and an annular extension 20 extending from upper body 12 about the axis of the cannular space 18. Base 14 is provided with a cylindrical cavity 22 formed for receiving said annular extension 20. As best shown in Fig. 2, the inner surface of the wall defining cylindrical cavity 22 is provided with an annular lip 24 that defines an opening to the lower chamber 26 formed in the surface of base 14. The outer surface of annular extension 20 frictionally engages the inner surface of the cylindrical cavity wall. In the illustrated embodiment, the outer surface of annular extension 20 is provided with screw threads 30 for engaging corresponding screw threads 32 located on the inner surface of the cylindrical cavity wall.

Substrate interface 16 comprises a layer of submucosal tissue. The submucosal tissue layer can comprise several different forms of submucosal tissue including, but not limited to, intact submucosal tissue-coated porous surfaces, soluble submucosal tissue-coated porous surfaces, or intact submucosal tissue alone. One preferred porous surface for use in accordance with the



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present invention is a polycarbonate filter. Substrate interface 16 is formed to have a diameter approximately equal to that of cylindrical cavity 22 so that upon insertion of substrate interface 16 into cylindrical cavity 22, substrate interface 16 will contact annular lip 24 to provide an upper boundary for lower chamber 26.

The annular extension 20 of upper body 12 has a length sufficient to allow the end portion 40 of annular extension 20 to contact substrate interface 16 when substrate interface 16 is positioned on lip 24 and annular extension 20 is inserted into cylindrical cavity 22. Thus an annular portion of substrate interface 16 seated on annular lip 24 is pressed against annular lip 24 by end portion 40 of annular extension 20, thus securing the substrate interface between the annular lip 24 and the annular extension 20. Accordingly, substrate interface 16 in cooperation with the annular space of annular extension 20 defines upper chamber 34 when substrate interface 16 and annular extension 20 are inserted into cylindrical cavity 22 (See Fig. 2).

Liquid media is introduced into the lower chamber 26 (prior to insertion of substrate interface 16 and annular extension 20 into cylindrical cavity 22) and upper chamber 34 (after the insertion of substrate interface 16 and annular extension 20 into cylindrical cavity 22) to provide nutrients for eukaryotic cell growth. Alternatively the invasion chamber can be provided with means for accessing the first and second chambers after assembly of the device to allow the addition and removal of liquids. A chemoattractant can optionally be added to the lower chamber 26 to promote invasion of the cell culture into substrate interface 16.

In another embodiment of the present invention, cell growth substrates in accordance with the present invention are formed from fluidized forms of submucosal

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tissue. The fluidized submucosal tissue can be gelled to form a solid or semi-solid matrix. Eukaryotic cells can then be seeded directly on the surface of the matrix and cultured under conditions conducive to eukaryotic cell proliferation.

The cell growth substrate of the present invention can be combined with nutrients, including minerals, amino acids, sugars, peptides, proteins, or glycoproteins that facilitate cellular proliferation, such as laminin and fibronectin and growth factors such as epidermal growth factor, platelet-derived growth factor, transforming growth factor beta, or fibroblast growth factor. In one preferred embodiment fluidized or powder forms of submucosal tissue can be used to supplement standard eukaryotic culture media to enhance the standard media's capacity for sustaining and inducing the proliferation of cells cultured *in vitro*.

In accordance with the present invention there is provided a cell culture composition for supporting growth *in vitro* of an eukaryotic cell population in combination with submucosal tissue of a warm-blooded vertebrate. The composition comprises nutrients, and optionally growth factors required for optimal growth of the cultured cells. The submucosa substrates of the present invention can be used with commercially available cell culture liquid media (both serum based and serum free). When grown in accordance with this invention, proliferating cells can either be in direct contact with the submucosal tissue or they can simply be in fluid communication with the submucosal tissue. It is anticipated that the cell growth compositions of the present invention can be used to stimulate proliferation of undifferentiated stems cells as well as differentiated cells such as islets of Langerhans, hepatocytes and chondrocytes. Furthermore the described cell growth composition is believed to support the growth

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of differentiated cells while maintaining the differentiated state of such cells.

It has been well documented that submucosal tissue is capable of inducing host tissue proliferation, remodeling and regeneration of appropriate tissue structures upon implantation in a number of microenvironments *in vivo* (e.g., tendon, ligament, bone, articular cartilage, artery, and vein). The use of such tissue in sheet form and fluidized forms for inducing the formation of endogenous tissues is described and claimed in U.S. Patent Nos. 5,281,422 and 5,275,826, the disclosures of which are expressly incorporated by reference.

In another embodiment of the present invention the tissue replacement capabilities of graft compositions comprising submucosal tissue of warm-blooded vertebrates are further enhanced or expanded by seeding the tissue with various cell types, prior to implantation. For example, submucosal tissue may be seeded with endothelial cells, keratinocytes, or islet of langerhans cells for use as a vascular graft, skin replacement, or auxiliary pancreas, respectively. Alternatively, the submucosal tissue can be seeded with mesenchymal cells (stem cells) initially for expansion of the cell population and thereafter for implantation into a host. Submucosal tissue can also serve as a delivery vehicle for introducing genetically modified cells to a specific location in a host. The submucosal tissue for use in accordance with this embodiment can either be in a fluidized form or in its native solid form. Optionally, after the submucosal tissue has been seeded with eukaryotic cells, the graft composition can be subjected to conditions conducive to the proliferation of eukaryotic cells to further expand the population of the seeded cells prior to implantation of the graft into the host.

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In one embodiment, compositions comprising submucosal tissue and a proliferating cell population can be encapsulated in a biocompatible matrix for implantation into a host. The encapsulating matrix can be configured to  
5 allow the diffusion of nutrients to the encapsulated cells while allowing the products of the encapsulated cells to diffuse from the encapsulated cells to the host cells. Suitable biocompatible polymers for encapsulating living cells are known to those skilled in the art. For example a  
10 polylysine/alginate encapsulation process has been previously described by F. Lim and A. Sun (Science Vol. 210 pp. 908-910). Indeed, vertebrate submucosa itself could be used advantageously to encapsulate a proliferating cell population on a submucosal matrix in accordance with this  
15 invention for implantation as an artificial organ.

Submucosal tissue advantageously provides a physiological environment that supports the differentiation of cells cultured *in vitro* on the submucosal tissue. Thus, cell culture substrates comprising submucosal tissue can be  
20 used in combination with standard cell culture techniques known to those of ordinary skill in the art, to produce tissue grafts, *in vitro*, for implantation into a host in need thereof. The cells of such a tissue perform their proper natural function based on cell type and position  
25 within the submucosal tissue graft construct.

The method of forming a tissue graft *in vitro* comprises the steps of seeding eukaryotic cells onto a cell growth substrate comprising submucosal tissue of a warm-blooded vertebrate and culturing the cells *in vitro* under  
30 conditions conducive to proliferation of the eukaryotic cells. Advantageously the synthesis *in vitro* of a tissue graft construct, wherein the cells of the tissue perform their proper natural function, allows the generation of tissue grafts from an initially small cell population that  
35 can be expanded *in vitro* prior to implantation.

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## Example 1

Sterilization of Submucosal Tissue

Because cell culture techniques must be performed under strict aseptic conditions, if antibiotics are not included in the culture system, the submucosa tissue must be prepared in a sterile manner for use as a cell culture substrate. Numerous sterilization methods have been investigated to assess the effect of sterilization on the biotropic properties of submucosal tissue. Sterilization techniques which do not significantly weaken the mechanical strength and biotropic properties of the tissue are preferred. The following sterilization methods for intestinal submucosa have been evaluated: peracetic acid sterilization, 2.5 Mrad gamma-irradiation, 1.0 Mrad gamma-irradiation, Exspor (Alcide, Norfolk, CT) sterilization and various combinations of these sterilization methods. Gamma-irradiation was performed on hydrated submucosal tissue using a <sup>60</sup>Cobalt-gamma chamber. Exspor sterilization was performed according to manufacturer's specifications using a sterilant volume (ml) to intestinal submucosa (g) ratio of 10 to 1.

Various cell types (e.g., IMR-90, FR, HT-29, RPEC) were seeded upon the sterilized submucosa and their growth characteristics were analyzed at 1,3,7 and 14 days. Results obtained for all cell types showed that submucosal derived growth substrates sterilized by gamma irradiation or peracetic acid treatments supported some degree of adherence and growth of cells. However, cells seeded onto peracetic acid sterilized submucosal derived substrates showed increased adherence, increased survival, and enhanced rates of proliferation and differentiation; peracetic acid appears to be the preferred sterilization technique for preparation of submucosa as a cell culture substrate.

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## Example 2

Sterilization of Submucosal Tissue with Peracetic Acid

Submucosal tissue is soaked in a peracetic acid/ethanol solution for 2 hours at room temperature using a ratio of 10:1 (mls peracetic solution: grams submucosal tissue) or greater. The peracetic acid/ethanol solution comprises 4% ethanol, 0.1% (volume: volume) peracetic acid and the remainder water. The 0.1% peracetic acid component is a dilution of a 35% peracetic acid stock solution commercially available and defined as in table 1. Preferably, the submucosal tissue is shaken on a rotator while soaking in the peracetic acid solution. After two hours, the peracetic acid solution is poured off and replaced with an equivalent amount of lactated Ringer's solution or phosphate buffered saline (PBS) and soaked (with shaking) for 15 minutes. The submucosal tissue is subjected to four more cycles of washing with lactated Ringer's or PBS and then rinsed with sterile water for an additional 15 minutes.

Table 1: Chemical Composition of the 35% Peracetic Acid Solution

Composition, % by weight		
	Peracetic acid	35.5
25	Hydrogen peroxide	6.8
	Acetic acid	39.3
	Sulfuric acid	1.0
	Water	17.4
	Acetyl peroxide	0.0
30	Stabilizer	500 PPM

## Typical active oxygen analysis, % by weight

	Active Oxygen as peracid	7.47
	Active Oxygen as H <sub>2</sub> O <sub>2</sub>	2.40
35	Total active oxygen	10.67

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## Example 3

Growth Characteristics Of Various Cell Types On Sterilized Submucosa

5 Small intestinal submucosa was harvested and prepared from freshly euthanatized pigs as described in U.S. Patent No. 4,902,508. Following sterilization via various techniques (gamma irradiation, peracetic acid, etc.), the submucosal tissue was clamped within a  
10 polypropylene frame to create a flat surface area (50 mm<sup>2</sup>) for cell growth. The frame was submerged in culture medium to allow access of medium nutrients to both surfaces of the submucosal tissue. Various cell types were seeded (3 x 10<sup>4</sup> cells/submucosal tissue section) on the submucosal tissue  
15 and then placed in a 5% CO<sub>2</sub>, 95% air incubator at 37°C. Following various periods of time, the seeded submucosal tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned (6 um). Various histological and immunohistochemical staining procedures were then  
20 applied to determine the cell growth characteristics.

To date, the growth characteristics of the following cell lines have been studied using submucosal tissue as a growth substrate:

25	CELL LINE	CELL LINE DESCRIPTION
	CHO	Chinese hamster ovary cells
	3T3	Swiss albino mouse embryo fibroblasts
	C3H10T1/2	C3H mouse embryo, multi-potential
30	FR	Fetal rat skin (Sprague Dawley)
	IMR90	Human fetal lung fibroblasts
	HT-29	Human colon adenocarcinoma, moderately well differentiated, grade II
	RPEC	Rat pulmonary endothelial cells
35	HUVEC	Human umbilical vein cells
	SCC-12	Squamous Cell Carcinoma

Table 2 summarizes various cell types and the corresponding specific medium conditions used to culture on  
40 the submucosa derived cell culture substrates. The medium

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chosen represents optimal or near optimal conditions for propagation of each cell type under standard cell culture conditions (i.e., plastic tissue culture flasks). All cell preparations were incubated at 37° C in a humidified atmosphere of 5% CO<sub>2</sub>/air.

**Table 2: Cell types and corresponding culture conditions investigated using Intestinal Submucosal Tissue as a cell growth matrix**

10

	CELL TYPE	MEDIUM
	3T3 (American Type Culture Collection (ATCC), CRL 1658) Swiss mouse embryo fibroblasts	DMEM (Dulbecco's modified Eagle's medium) with 1.5 g/L NaHCO <sub>3</sub> , 10% NNCS (neonatal calf serum), 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine
15	FR (ATCC, CRL 1213) cell line developed from a skin biopsy of a fetal (18 day gestation) germ-free Sprague Dawley rate	DMEM, 10% NNCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine
20	HT-29 (ATCC, HTB 38) cell line derived from human colon adenocarcinoma	McCoy's, 10% NNCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine
	HUV-EC-C (ATCC, CRL 1730) endothelial cell line isolated from human umbilical vein	F12 K medium, 10% FBS (fetal bovine serum), 100 ug/ml heparin, 50 ug/ml endothelial cell growth supplement, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine
25	IMR-90 (ATCC, CCL 186) human diploid fibroblasts	McCoy's 5A medium, 20% NNCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine
30	RPEC (J.P. Robinson, Purdue University) endothelial cell line derived from rat pulmonary endothelial cells	RPMI 1640, 5% NCS (newborn calf serum) 5% FBS (fetal bovine serum), 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine
	C3H10T1/2 (ATCC, CCL 226) mouse embryo fibroblasts	BME (basal medium Eagle), 10% FBS, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamin
35	SCC-12 (W. Greenlee, Purdue University) squamous cell carcinoma	DMEM, 5% FBS (fetal bovine serum), 4 mM L-glutamine, 1 mM sodium pyruvate
	CHO (Chinese Hamster Ovary Cells)	F12 Medium 10% FBS with antibiotics (Neomycin)



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The cellular growth on both the luminal and abluminal sides of intestinal submucosal tissue has been investigated. Intestinal submucosal tissue as a growth substrate exhibits sidedness; that is, the cell/matrix interactions are different when the cells are cultured on the abluminal versus the luminal side of the intestinal submucosal tissue. When selected cell types, such as rat FR cells are seeded on the luminal side, the cells attach to the matrix surface and proliferate to form a cellular polylayer. Alternatively, when FR cells are seeded on the abluminal side, the cells not only grow along the surface but also migrate into the submucosal matrix.

The stratum compactum of the luminal side of vertebrate intestinal submucosal tissue provides a dense connective tissue matrix and more readily supports monolayer or polylayer formation of select cell types (i.e. endothelial and epithelial cells). Alternatively, the abluminal side represents a more loose connective tissue structure that more readily supports migration of cells within the matrix structure (i.e. fibroblasts).

IMR-90 fibroblasts, when seeded upon the abluminal or luminal sides of the intestinal submucosal tissue, quickly became adherent and proliferated throughout the matrix components. These cells illustrated their characteristic spindle shape and large vesicular nucleus within the extracellular matrix components. However, 3T3 fibroblasts showed minimal adherence and growth potential when seeded upon the intestinal submucosal tissue.

Endothelial cells formed a confluent monolayer of spindle-shaped cells along the stratum compactum surface of the intestinal submucosal tissue within 3 days. At later times the monolayer became more dense and some cells intercalated down into the matrix components. Interestingly, some endothelial cells that penetrated into the matrix components formed a lining along the lumen of

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structures representing original blood vessels of the native intestine.

To date, the growth characteristics of the following primary cell strains have been studied using  
5 intestinal submucosal tissue as a growth substrate:

#### CELL STRAIN

Rat Cardiac Muscle  
Porcine Smooth Muscle (aorta)  
10 Porcine Endothelial (aorta)  
Rabbit Smooth Muscle (aorta)  
Rabbit Endothelial(aorta)  
Porcine Smooth Muscle and Endothelial (mixed & co-cultured)  
15 Human Osteoblasts  
Human Endothelial Cells

Primary cell strains are cells that have been harvested from an organism and placed in culture.  
20 Subsequent passages of these cells (from 2-3 times) using standard *in vitro* cell culture techniques (to increase the number of cells) were frozen for later use. Each of the above listed cell strains was thawed, cultured in the presence of intestinal submucosal tissue and examined  
25 histologically. Each of the cultured cell strain populations proliferated and retained their differentiated appearance as determined by histological examination. For example, after 7-14 days of culture on intestinal submucosal tissue: the human osteoblast cells continued to  
30 accumulate appatite crystals and respond to osteogenic stimuli such as hormones; rat cardiac muscle cells retained their contractile properties; porcine smooth muscle cells retained smooth muscle actin; and porcine endothelial cells made factor eight.

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## Example 4

Intestinal Submucosal Cell Culture Substrates as a Tumor  
Cell Growth Model System

The morphology and invasive properties of an established cell line from a human squamous cell carcinoma of the face known as SCC-12 (obtained from W. Greenlee, Purdue University) cultured *in vitro* were studied. When grown under standard cell culture conditions for skin cells (e.g., gamma-irradiated or mitomycin C-treated feeder layer of Swiss 3T3 mouse fibroblasts) a monolayer of flattened cells is formed. However SCC-12 cells when seeded upon the abluminal surface of intestinal submucosal tissue, showed, upon histological examination active degradation of the submucosal matrix components and invasion of the intestinal submucosal tissue.

SCC-12 cells were seeded ( $3 \times 10^4$  cells/ $0.8 \text{ cm}^2$  of intestinal submucosal tissue) on either the abluminal or luminal surface of sterilized intestinal submucosal tissue and floated in growth medium consisting of DMEM containing 5% fetal calf serum, 4mM L-glutamine, and 1mM sodium pyruvate. At timepoints representing 3, 7, 14, and 21 days, the growth characteristics were analyzed using standard histologic techniques. On day 3, the cells were strongly adherent and appeared to form a continuous layer (1-2 cells thick) along surface of the intestinal submucosal tissue. Morphologically, the cells were round and actively producing extracellular matrix products. After 7 days, a significant difference was noted in the cells' ability to invade the abluminal versus the luminal surface of the intestinal submucosal tissue. The layer of cells along the luminal surface of the intestinal submucosal tissue appeared to only increase in density. Alternatively, those cells seeded upon the abluminal surface, showed active degradation of the submucosal matrix components and penetration up to 30  $\mu\text{m}$ . At longer

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5 durations, there was an increasing number of cells at greater depths of penetration and a greater extent of intestinal submucosal tissue degradation. Although the SCC-12 cells actively invade intestinal submucosal tissue from both the abluminal and luminal surfaces, the observed invasion rate was greater when SCC-12 cells were placed on the abluminal side.

#### Other Metastatic and Non-metastatic Tumor Cell Lines

10 For these experiments small intestinal submucosa was sterilized with peracetic acid and clamped with a polypropylene frame to create a flat surface area for cell growth (50 mm<sup>2</sup>). The frame was submerged in culture medium to allow access of medium nutrients to both surfaces of the  
15 intestinal submucosal tissue. The cells were seeded (5 X 10<sup>4</sup> cells/0.8 cm<sup>2</sup> of intestinal submucosal tissue) and placed 5% CO<sub>2</sub>, 95% air incubator at 37°C. Following 3, 7, 10, and 14 days, the seeded submucosal tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and  
20 sectioned (5µm). Standard H&E histological staining was performed for morphological examination.

The growth characteristics of the following cell lines, cultured on intestinal submucosal tissue under the corresponding culture conditions, were investigated.

25

CELL TYPE	CULTURE MEDIUM
Ras-transformed NIH 3T3 fibroblasts, highly metastatic	Dulbecco's modified Eagle's Medium (DMEM), 5% Newborn Calf Serum, 100 U/ml penicillin, 100 ug/ml streptomycin
parental NIH 3T3 fibroblasts non-metastatic	DMEM, 10% Newborn Calf Serum, 100 U/ml penicillin, 100 ug/ml streptomycin

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canine prostate adenocarcinoma, metastatic	RPMI 1640, 10% Newborn Calf Serum, 100 U/ml penicillin, 100 ug/ml streptomycin
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5

When grown on intact intestinal submucosal tissue, the non-metastatic parental NIH 3T3 fibroblasts attached to the surface of the intestinal submucosal tissue but did not degrade or penetrate into the matrix. On the other hand, highly metastatic ras-transformed NIH 3T3 fibroblasts showed attachment, degradation, and aggressive penetration of the matrix. Likewise, a cell line established from metastatic canine prostate adenocarcinoma showed formation of glandular-like structures along the surface and focal regions of matrix degradation and migration. These growth and differentiation characteristics are similar to those exhibited naturally by these cells *in vivo*. In general, such characteristics are not observed when cells are grown on plastic.

15  
20

#### Use of Invasion Chambers to Assess Invasive Properties of Cells

The invasive properties of various cultured cells was investigated through the use of invasion chambers. The growth characteristics of the cultured cells grown on soluble submucosal tissue-coated polycarbonate filters was compared to cells grown on Matrigel-coated polycarbonate filters according to the following procedure.

Polycarbonate filters (13 mm, 8 um pore size) were coated with soluble submucosal tissue or Matrigel, air dried in a laminar flow hood, and reconstituted in serum-free medium. Serum free medium containing 25 ug/ml fibronectin was placed in the lower well of the Blind well chamber to serve as a chemoattractant. The coated filters were placed as an

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interface between the upper and lower wells of the invasion chamber. Cells (approximately  $2 \times 10^5$ ) suspended in serum-free medium containing 0.1% BSA were seeded upon the coated filters within each invasion chamber and the chamber  
5 incubated at 37°C in 5%CO<sub>2</sub>/95% air.

At timepoints ranging from 6 to 24 hours, the filters and associated substrate were collected, fixed in neutral buffered formalin, stained with 0.5% toluidine blue, and evaluated for invasiveness using light  
10 microscopy. As observed with Matrigel, submucosal tissue supported the attachment, degradation, and migration of metastatic tumor cells (ras-transformed NIH 3T3 fibroblasts) *in vitro*. Alternatively, non-metastatic cells (parental NIH 3T3 fibroblasts) showed minimal to no  
15 migration on either Matrigel or submucosal tissue coated filters. Analysis of the growth characteristics of the cultured cells can also be conducted by labeling the cells using various methods including radioactive or fluorescent probes to facilitate quantitation of invasiveness.  
20

#### Example 5

##### Intestinal Submucosal Tissue Supports Cytodifferentiation

FR Epithelial cells form a stratified polylayer when cultured on the luminal (stratum compactum) side of  
25 intestinal submucosal tissue. Cells adjacent to the intestinal submucosal tissue were columnar in shape and became progressively more flattened near the surface of the polylayer. After 14 days, structures resembling desmosomes were identified and the cellular layer stained positively  
30 for cytokeratin with a pan cytokeratin antibody. In addition, it appeared that the epithelial cells produced supporting matrix products (potentially basement membrane) as they do *in vivo* under normal healthy conditions. These findings suggest that the intestinal submucosal tissue

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supports natural epithelial cell maturation and differentiation processes.

The observed stratification of FR cells grown on the luminal side (stratum compactum) of a submucosal growth substrate provides evidence that the intestinal submucosal tissue supports and induces cellular differentiation in vitro. To verify the induction of cytodifferentiation of the FR cells, immunohistochemical and immunofluorescence analyses were performed for detecting the production of cytokeratin by FR cells cultured in the presence and absence of intestinal submucosal tissue. Cytokeratin is a predominant intracellular structural protein produced by terminally differentiated epithelial cells known as keratinocytes. Immunohistochemistry was performed on the protease-digested, formalin-fixed, paraffin embedded sections of FR cells grown on intestinal submucosal tissue using an anti-pan cytokeratin (C2931, Sigma, St. Louis, MO) as the primary antibody. Immunodetection was performed using the avidin-biotin complex (ABC) method and the Biogenex supersensitive StriAvisGen kit (Vector Laboratories, Burlingame, CA). Tissue sections representing rat skin biopsies and HT29 cells grown on intestinal submucosal tissue were included in the analysis as positive and negative controls, respectively.

Results indicated a gradation of cytokeratin staining along the FR cellular polylayer with those cells at the surface of the polylayer staining most intensely. A similar positive staining pattern was observed in the cells forming the epidermal layer of the rat skin. However, no cytokeratin was detected in the specimens representing HT29 cells cultured on intestinal submucosal tissue.

An immunofluorescence analysis for cytokeratin was performed using flow cytometry to determine if the FR cell line expressed the differentiation product cytokeratin under standard culture conditions (in the absence of

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intestinal submucosal tissue). Swiss 3T3 Fibroblast (3T3) and squamous cell carcinoma (SCC-12) cell lines were included in the analysis as negative and positive controls respectively. Cells were harvested from tissue culture  
 5 flasks, permeabilized using a cold methanol pretreatment, and incubated in the presences of anti-pan cytokeratin antibody at various dilutions (including the absence of anti-pan cytokeritin antibody to serve as a control). A  
 10 goat anti-mouse antibody conjugated with fluorescein isothiocyanate (GAM-FITC) was then applied to facilitate immunodetection. The cell preparations were then analyzed on a EPICS Elite flow cytometer (Coulter Corp., Hialeah, FL) using 488 nm excitation produced by an air-cooled argon  
 15 laser. Fluorescence emissions were measured at 525 nm with a bandpass filter. Untreated cells and cells treated only with GAM-FITC were also analyzed to establish background fluorescence levels. Table 3 represents the relative  
 20 percentage of FITC fluorescence for each cell type following indirect immunofluorescence staining. As the data indicate only the positive control SCC-12 cell line expresses cytokeratin and the FR cell line does not express cytokeratin under standard culture conditions in the absence of submucosal substrate.

25           **Table 3 Indirect Immunofluorescence Analysis for Cytokeratin SCC-12, 3T3 and FR Cells**

	<u>Cell Type</u>	<u>Dilution of Anti-Pan Cytokeratin</u>	<u>Percent GAM-FITC Fluorescence</u>
30	SCC-12	0(control)	2%
	SCC-12	1:100	72%
	SCC-12	1:400	74%
	SCC-12	1:1000	76%
	SCC-12	1:4000	72%
35	3T3	0(control)	11%
	3T3	1:100	10%
	3T3	1:400	18%
	3T3	1:1000	8%
	3T3	1:4000	5%
40	FR	0(control)	6%
	FR	1:100	11%



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	<u>Cell Type</u>	<u>Dilution of Anti-Pan Cytokeratin</u>	<u>Percent GAM-FITC Fluorescence</u>
	FR	1:400	6%
	FR	1:1000	4%
5	FR	1:4000	4%

## Example 6

Isolation Of Hamster Pancreatic Islets

Hamster pancreatic islets were isolated as previously described by Gotoh et al. (Transportation Vol. 43, pp. 725-730(1987)). Briefly, 6-8 week old Golden hamsters (Harlan, Indianapolis, IN) were anesthetized via inhalation of Metofane (Methoxyflurane; Pitman-Moore; Mundelein, IL). The common bile duct was cannulated under a stereomicroscope with a polyethylene catheter (PE-10 tubing; CMS; Houston, TX), through which approximately 3-4 mls of ice cold M-199 medium (commercially available from Gibco BRL) containing 0.7 mg/ml of collagenase P was injected slowly until whole pancreas was swollen. The pancreas was excised and digested at 37° C for approximately 50 minutes in M-199 medium containing 100 µg/ml of penicillin G and 100 µg/ml of streptomycin (no additional collagenase). The digest was washed three times in ice cold M-199 medium and passed sequentially through a sterile 500 µm stainless steel mesh, then a 100 µm mesh. Following purification by centrifugation through a ficoll density gradient (1.045, 1.075, 1.085 and 1.100) at 800 g for 10 min, islets were recovered from the top two interfaces.

Culturing of Pancreatic Islet Cells on IntestinalSubmucosal Tissue

Islets of Langerhans (islet cells) were cultured on submucosal cell growth substrates at 37°C in an incubator supplemented with 5% CO and 95% air. The islet cells were cultured in the presence of various forms of

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intestinal submucosal tissue using the following procedures:

1. Direct Contact: Intestinal submucosal tissue and the cultured cells physically contact one another.
- 5 2. Indirect Contact: Intestinal submucosal tissue and the cultured cells are separated by a stainless steel mesh.
3. Solubilized intestinal submucosal tissue is added to the culture media
- 10 4. Cells are cultured on solubilized intestinal submucosa coated culture plate. The coating was applied by placing 1 ml of solubilized intestinal submucosal tissue in a 35mm culture plate, heated at 37°C for 2 hours, removing the excess intestinal submucosal tissue fluid by aspiration
- 15 and washing the coated plates once with culture media.

In direct contact culture method, an intestinal submucosa membrane of approximately 1x1 cm was placed on top of stainless steel mesh with the stratum compactum side facing up. Isolated islets were then placed onto the

20 membrane and continuously cultured in M-199 medium (commercially available from Gibco BRL) for 7 days. Cell proliferation was examined every second day under a stereomicroscope and was compared with the control group (cultured in the absence of submucosa tissue).

25

#### Sterilization of Submucosal Tissue Before Co-culturing

1. Intestinal submucosal tissue derived cell culture substrates were sterilized by several different means: peracetic acid treatment or gamma irradiation.
- 30 Gamma irradiated and the native (no further treatment after isolation of the intestinal submucosal tissue) membranes can be used directly as cell culture substrates provided they have been sufficiently rehydrated with the culture media prior to the co-culture (native membranes must be
- 35 cultured in the presence of antibiotics). Peracetic acid

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sterilized membranes, must first be washed to remove residual peracetic acid prior to culturing since peracetic acid residue may be toxic to the cells. Typically peracetic acid sterilized tissues were soaked in a large  
5 quality of medium for 24 hours followed by extensive washing with the same medium.

2. Solubilized forms of intestinal submucosal tissue were sterilized by dialyzing against 6.5% chloroform in either 0.1M acetic acid (AA-submucosa) or phosphate  
10 buffered saline (PBS-submucosa) for 2 hours at room temperature. The exterior surface of the dialysis tubing is sterilized by rinsing the outside of the tubing with 70% alcohol prior to removal of the intestinal submucosal tissue. The dialysis tubing has a molecular weight cut-off  
15 of 12,000-14,000; thus, proteins retained inside tubing are those with molecular weight greater than 14,000.

### Results

In the control group (islets cultured in the  
20 absence of submucosa tissue) examination of seven day cultures revealed that fibroblast cells had overgrown the islet cells.

When islet cells were cultured on growth substrates comprising intestinal submucosal tissue,  
25 overgrowth of the islet cells by fibroblast cells did not occur. In intestinal submucosal tissue direct culture systems, the islets became loosely packed with many cells surrounding the islet capsule. Cells migrated from the capsule and cell proliferation occurred on top of the  
30 membrane in the absence of fibroblast overgrowth. Culturing islet cells on intestinal submucosal tissue coated culture ware also appeared to facilitate migration of epithelioid cells out of the islet capsule. Further attachment to the coating surface and the formation of a  
35 monolayer of epithelioid cells was observed.

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These data indicate that submucosal substrates can be used to stimulate growth of islet cells *in vitro* without overgrowth of fibroblast cells. Islet cells can thus be isolated from pancreatic tissue and grown *in vitro*  
5 in contact with a cell growth substrate comprising intestinal submucosal tissue of a warm-blooded vertebrate under conditions conducive to the proliferation of the islet cells and without concurrent growth of fibroblasts. These islet cell culture compositions remain substantially  
10 free of fibroblast overgrowth.

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## CLAIMS:

1. A method for supporting the growth and differentiation of eukaryotic cells *in vitro*, said method comprising the step of contacting the cells *in vitro* with a cell growth substrate comprising submucosal tissue of a warm-blooded vertebrate under conditions conducive to the proliferation of said cells.

2. The method of claim 1, wherein the submucosal tissue is intestinal submucosa comprising the tunica submucosa delaminated from the tunica muscularis and at least the luminal portion of the tunica mucosa.

3. The method of claim 1 wherein the step of contacting the cells with a cell growth substrate comprises culturing the cells on culture ware that has been coated with fluidized submucosal tissue.

4. The method of claim 1, wherein the step of contacting the cells with a cell growth substrate comprises adding fluidized submucosal tissue to liquid cell culture media.

5. A culture medium composition for supporting the growth of a cell population, said composition comprising

submucosal tissue of a warm-blooded vertebrate; and nutrients to support growth of said cell population.

6. The composition of claim 5, wherein the submucosal tissue is intestinal submucosa comprising the tunica submucosa delaminated from the tunica muscularis and at least the luminal portion of the tunica mucosa.

7. The composition of claim 5, wherein the submucosal tissue is fluidized submucosal tissue.

8. The composition of claim 5, wherein the submucosal tissue is digested with a protease for a period

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of time sufficient to solubilize the tissue and provide a substantially homogeneous solution.

9. A cell culture composition comprising submucosal tissue of a warm-blooded  
5 vertebrate;  
nutrients to support growth of said cell population and;  
a proliferating population of a preselected cell type.

10 10. The cell culture composition of claim 9 wherein the submucosal tissue is intestinal submucosal tissue.

11. The cell culture composition of claim 9 wherein the preselected cell type is islet cells  
15 substantially free of fibroblast overgrowth.

12. A method for enhancing the functional properties of submucosal tissue of a warm-blooded vertebrate as an implanted or injected tissue graft material, said method comprising the steps of seeding the  
20 submucosal tissue with cells of a preselected cell type prior to implanting or injecting the graft material into a host.

13. The method of claim 12 further comprising the step of subjecting the graft material to conditions  
25 conducive to the proliferation of the preselected cell type and proliferating the cells prior to implanting or injecting the graft material into the host.

14. A method for growing islet cells *in vitro* without concurrent growth of fibroblasts said method  
30 comprising the step of

contacting islet cells *in vitro* with a cell growth substrate comprising submucosal tissue of a warm-blooded vertebrate under conditions conducive to the proliferation of said cells.

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15. A method for analyzing *in vitro* the growth characteristics of tumor cells under variable tumor cell growth conditions, said method comprising the steps of
- 5        seeding said tumor cells on a cell growth substrate comprising submucosal tissue of a warm-blooded vertebrate and providing a culture medium containing nutrients necessary to the proliferation of said cells;
- selecting a variable cell growth condition;
- culturing the tumor cells on said substrate
- 10    in said nutrient medium under the selected condition, and;
- histologically examining the mucosal tissue substrate and the tumor cell population on said substrate.
16. The method of claim 15 wherein the selected growth condition is the presence or concentration of a
- 15    tumor cell growth modifier compound in the nutrient medium.
17. The method of claim 15 wherein the selected growth condition is a quantity of electromagnetic radiation.
18. A method for analyzing, *in vitro*, tumor
- 20    invasion of extracellular matrices, said method comprising the steps of
- seeding said tumor cells on a cell growth substrate comprising submucosal tissue of a warm-blooded vertebrate;
- 25        culturing the tumor cells on said substrate under conditions conducive to the proliferation of eukaryotic, and;
- histologically examining the mucosal tissue substrate and the tumor cell population on said substrate.
- 30    19. The method of claim 18 further comprising the step of altering one or more growth condition to determine the effect the alteration has on the growth characteristics of the tumor cells.
20. The method of claim 19 wherein the step of
- 35    altering one or more growth condition comprises the

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addition or change in concentration of a tumor cell growth modifier compound.

21. The method of claim 19 wherein the step of altering one or more growth condition comprises exposing  
5 the tumor cells to a quantity of electromagnetic radiation.

22. A device for studying the *in vitro* invasive growth properties of cells, said device comprising  
an upper body defining a first chamber;  
a base defining a second chamber;  
10 a substrate interface separating said first and second chambers, and;

means for biasing the upper body toward the base to hold the substrate between the upper body and the base, wherein the substrate interface comprises submucosal tissue  
15 of a warm-blooded vertebrate tissue.

23. The device of claim 22, wherein the substrate interface consists essentially of submucosal tissue.

24. The device of claim 22, wherein the  
20 substrate interface comprises submucosal tissue-coated filters.

25. The device of claim 22, wherein the first and second chambers are filled with nutrient media.

26. The device of claim 22, wherein,  
25 said upper body comprises an axially extending projection having a bore extending through, and parallel to, the axially extending projection, wherein the axially extending projection provides a first annular surface;  
said base comprising an end wall defining the  
30 opening to the second chamber, wherein the end wall provides a second annular surface, and said first and second annular surfaces being substantially complementary in shape, and;



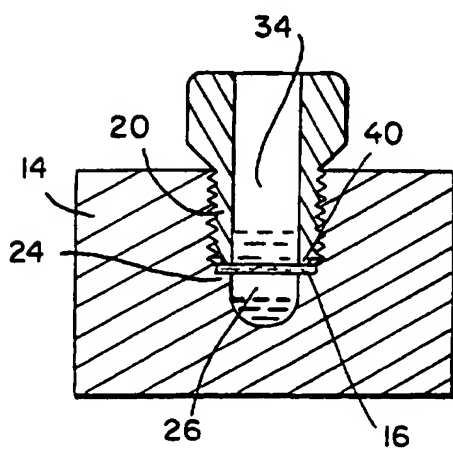
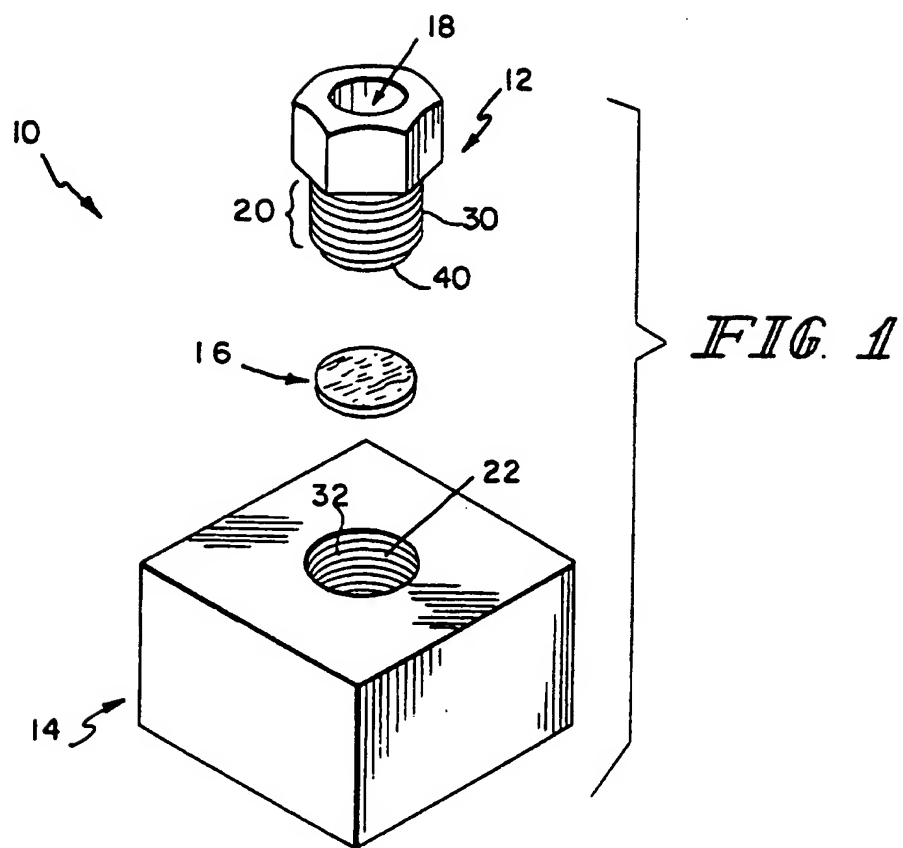
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means for biasing the first annular surface towards the second annular surface to hold said substrate interface between said first and second annular surface.

27. The invasion chamber of claim 26, wherein  
5 the submucosal tissue layer consists essentially of submucosal tissue.

28. The invasion chamber of claim 26, wherein the submucosal tissue layer comprises submucosal tissue-coated porous support.

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## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US96/01842
**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 5/00; A61K 35/37, 35/38; C12M 3/00

US CL : 435/240.1, 240.2, 240.23, 240.243, 240.3, 284; 424/551;

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.1, 240.2, 240.23, 240.243, 240.3, 284; 424/551;

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US, 5,281,422, A (BADYLAK ET AL.) 25 January 1994, column 3, lines 18-28.	5, 6, 9, and 10 ----- 1-8, 23, 24, 27, and 28
X ---- Y	KUO, et al. Biohybrid Islet-Gland Equivalent for Transplantation. Journal of Cellular Biochemistry. February 1994, Supplement 18C, abstract #PZ110. See the entire reference.	5, 6, 9-11, and 14 ----- 1-8, 23, 24, 27, and 28
Y	KLEINMAN et al. Basement Membrane Complexes with Biological Activity. Biochemistry. 1986, Vol. 25, Nol. 2, pages 312-318, especially pages 313 and 316.	1-8, 23, 24, 27, and 28



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

21 MAY 1996

Date of mailing of the international search report

10 JUN 1996

 Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/01842

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BADYLAK et al. Directed Connective Tissue Remodelling upon a biologic Collagen Substrate. Tissue Engineering, #CE027, page 124. See entire reference.	1-8, 23, 24, 27, and 28
Y	DEMAIN, ET AL. Manual of Industrial Microbiology and Biotechnology. Washington, D.C.: American Society for Microbiology, 1986, Chapter 20, pages 248-262, see entire document.	1-8, 22-28
Y	FRESHEY, Culture of Animal Cells--A Manual of Basic Technique. New York: Alan R. Liss, Inc., Chapter 12, pages 119-143, see entire document.	1-8
Y	SIGMA CELL CULTURE CATALOGUE, 1994, p. 160.	22-28
Y	KASHTAN, H. et al. Intra-Rectal Injection of Tumour Cells: A Novel Animal Model of Rectal Cancer. Surgical Oncology. June 1992, Vol. 1, No. 3, pages 251-256. See entire document.	15-21
Y, P	US, 5,478,739 A (SLIVKA ET AL.) 26 December 1995, see entire document.	15-21
Y, E	US, 5,518,915 A (NAUGHTON ET AL.) 21 May 1996, see entire document.	15-21
Y	US, 4,743,552 A (FRIEDMAN ET AL.) 10 May 1988, see entire document.	15-21
Y	EL HOUSSEINY, I.I. et al. Orthotopic Implantation of Primary N-[4-(5-Nitro-2-furyl)-2-thiazoiyl]formamide-induced Bladder Cancer in Bladder Submucosa: An Animal Model for Bladder Cancer Study. Cancer Research. February 1983, Vol. 43, pages 617-622. See entire document.	15-21

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/01842

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☒ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/01842

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-11 and 14, drawn to a method for supporting the growth and differentiation of eukaryotic cells in vitro, cell culture medium compositions, and a method for growing islet cells in vitro.

Group II, claims 12 and 13, drawn to a method for enhancing the functional properties of submucosal tissue.

Group III, claims 15-17, drawn to methods for analyzing, in vitro, the growth characteristics of tumor cells.

Group IV, claims 18-21, drawn to a method for analyzing, in vitro, tumor invasion of extracellular matrices.

Group V, claims 22-28, drawn to a device for studying the in vitro invasive growth properties of cells.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the groups are directed to independent methods, media compositions, and a device that require distinct process steps and ingredients. For example, Group II requires enhancement of the properties of a graft material in vivo whereas the other groups are directed to in vitro methods and compositions. Group III requires the additional steps of comparatively analyzing tumor cell growth and selection of a growth condition. Components such as electromagnetic radiation are only needed to practice the inventions of Groups III and IV. Group V is directed to a device used to study the in vitro invasive growth properties of cells. No other group is directed to the invasive growth properties of cells. Group IV is directed to invasive properties, but of tumor cells in extracellular matrices.

The search for each of the above inventions is not co-extensive particularly with regard to the literature search. Further, a reference which would anticipate the invention of any one group would not necessarily anticipate or even make obvious any other group.

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